Cell type-dependent proapoptotic role of Bcl2L12 revealed by a mutation concomitant with the disruption of the juxtaposed *Irf3* gene

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The generation of mice lacking the expression of the IRF3 transcription factor (*Irf3*$^{-/-}$ mice) has revealed its crucial role in the activation of the type I IFN response. The *Bcl2l12* gene, encoding Bcl2L12 protein structurally related to the Bcl-2 family, was found to almost overlap with the *Irf3* gene, and the null mutation previously introduced into the *Irf3* allele resulted in the functional inactivation of the *Bcl2l12* gene; therefore, the mice are correctly termed *Irf3*$^{-/-}$*Bcl2l12*$^{-/-}$ mice. Embryonic fibroblasts from *Irf3*$^{-/-}$ *Bcl2l12*$^{-/-}$ mice (*Irf3*$^{-/-}$*Bcl2l12*$^{-/-}$ MEFs) showed resistance to DNA damage-induced apoptosis, accompanied by impaired caspase cleavage. This apoptotic defect in *Irf3*$^{-/-}$*Bcl2l12*$^{-/-}$ MEFs was rescued by the ectopic expression of Bcl2L12, but not IRF3. The Bcl2L12-mediated apoptotic response depended on the cell type and extracellular stimulus. In contrast, the previously reported defect in the induction of type I IFN genes by nucleic acids in *Irf3*$^{-/-}$*Bcl2l12*$^{-/-}$ MEFs was rescued by expressing IRF3, but not Bcl2L12. Thus, our present study revealed, on the one hand, a cell type-dependent proapoptotic function of Bcl2L12 and, on the other hand, confirmed the essential role of IRF3 in type I IFN response.

apoptosis | Golgi | innate immunity | mitochondria

One of the hallmarks of the innate immune response is the activation of the type I IFN (namely, IFN-α and IFN-β) response that occurs upon cellular infection by pathogens such as viruses. IFN regulatory factor 3 (IRF3) plays an essential role in the evocation of the type I IFN response, acting as the transcriptional activator on IFN promoters [reviewed in ref. 1]. Briefly, IRF3 is ubiquitously expressed in various cell types and resides in the cytosol in an inactive form in normally growing cells. Upon viral or bacterial infection, IRF3 undergoes phosphorylation by the kinases TBK1 (for TANK-binding kinase 1) and IKKε/i (inhibitor of NF-κB kinase ε/i) activated by various pattern recognition receptors of the innate immune system, typically, the cytosolic RIG-I (retinoic acid-inducible gene I), MDA5 (melanoma differentiation-associated gene 5), DAI (DNA-dependent activator of IRFs), and the transmembrane Toll-like receptors (TLRs) 3 and 4 (1, 2). This results in the translocation of IRF3 into the nucleus where it activates the transcription of type I IFN genes, particularly *Ifnb* and *Ifna4* (1). The nonredundant role of IRF3 in the activation of the type I IFN response in cooperation with IRF7 has been demonstrated by the study of mice carrying a null mutation in the *Irf3* allele (*Irf3*$^{-/-}$ mice) (3, 4). On the other hand, evidence has also been provided that IRF3 may be involved in DNA damage-induced apoptosis. It has been shown that IRF3 translocates to the nucleus upon DNA damage (5), and that the overexpression of IRF3 causes an apoptotic response in a cultured cell line (6). However, the extent to which IRF3 contributes to DNA damage-induced apoptosis has not been rigorously examined.

Bcl2-like-12 (Bcl2L12) is a proline-rich protein that contains a BH2-like sequence with significant homology to BH2 domains in other pro- and antiapoptotic Bcl-2 family proteins, and is encoded by a gene located closely to the *Irf3* locus in both humans and mice (7). To date, still little is known about the biological function of Bcl2L12, and its role in the regulation of apoptosis still remains obscure or even controversial. It has been reported that Bcl2L12 binds and neutralizes caspase-7 in human glioblastoma cell lines, thereby functioning as an antiapoptotic factor (8), and that Bcl2L12 induces the expression of the gene encoding the small heat shock protein α-basic-crystallin, which binds to procaspase-3 to inhibit its activation (9). On the other hand, it has been shown that Bcl2L12 exhibits proapoptotic activity in a breast cancer cell line (10). Thus, it is interesting and important to examine how Bcl2L12 contributes to the regulation of apoptosis using the gene disruption approach.

In the course of our study on the potential role of IRFs in the regulation of apoptosis, we found that the *Bcl2l12* gene almost overlaps with the *Irf3* gene and that the null mutation previously introduced into the *Irf3* allele in mice resulted in the functional inactivation of the *Bcl2l12* gene. Thus, the mutant mice we previously generated are found to be doubly deficient for *Irf3* and *Bcl2l12* genes, hence should be correctly termed *Irf3*$^{-/-}$*Bcl2l12*$^{-/-}$ mice. Consequently, this unexpected "*killing 2 birds with 1 stone*" type gene disruption gave us the onus of, on the one hand, formally reassessing the contribution of IRF3 in the activation of the type I IFN response and, on the other hand, examining the contribution of Bcl2L12 in the regulation of apoptosis. Here, we show that IRF3, but not Bcl2L12, indeed functions as an essential transcription factor for the evocation of the type I IFN response. Furthermore, we demonstrate that Bcl2L12, but not IRF3, critically contributes to the induction of DNA damage-induced apoptosis that may involve the activation of caspases-2, 3, and 9, and the Golgi apparatus. We discuss the significance of our present findings in the context of the regulation of the type I IFN response and apoptotic pathways, respectively regulated by IRF3 and Bcl2L12.

Results

Disruption Status of the *Bcl2l12* Gene. The *Irf3*-null allele had been generated by deleting the proximal part of the *Irf3* coding region together with its upstream promoter region by homologous recombination (3). During the course of our study on the regulation of apoptosis by IRF3, we found, by sequence analysis of the genomic DNA from *Irf3*$^{-/-}$ cells, that an 801-bp fragment of DNA, a region that spans from the 2nd exon of *Bcl2l12* to the

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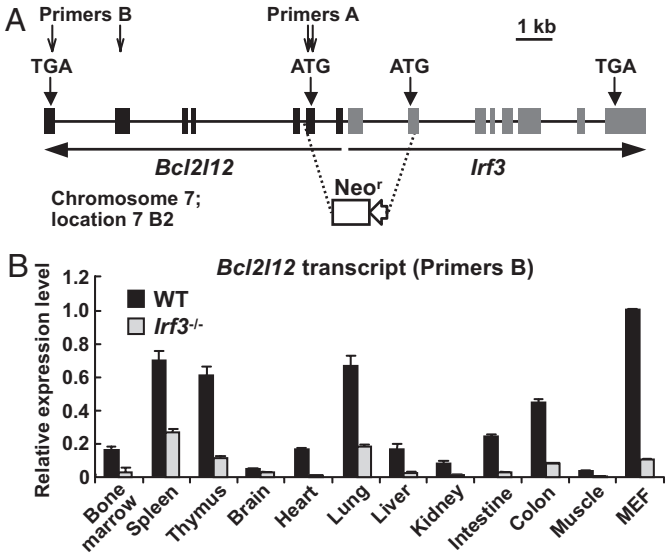

Fig. 1. Disruption of the *Bcl2l12* gene in *Irf3*-deficient mice. (*A*) Diagram of the gene locus encompassing *Bcl2l12* and *Irf3* genes at chromosome 7 in mice. The region replaced by the neomycin resistant gene cassette (Neo^r) in *Irf3*$^{-/-}$ (and therefore *Bcl2l12*$^{-/-}$) mice is depicted. (*B*) qRT-PCR for the *Bcl2l12* transcript in various tissues from WT and *Irf3*$^{-/-}$*Bcl2l12*$^{-/-}$ mice. Primers B targeting the 326th to 405th nucleotides from the AUG of the WT *Bcl2l12* mRNA were used. See Fig. S1 for data with primers A.

middle of the 2nd exon of *Irf3*, is absent and is replaced by the neomycin-resistant gene cassette (Fig. 1*A*). Therefore, unexpectedly, this genetic manipulation resulted in the partial deletion of the juxtaposed *Bcl2l12* gene, located on the opposite strand of the chromosome, because the 1st exons of the 2 genes are only 79 bp apart [Fig. 1*A*; (7)]. Thus, the transcription and translation start sites as well as the 5′ region, which likely contains the putative promoter region, of this gene are all deleted in the mutant mice.

We examined the mRNA expression status of the *Bcl2l12* gene in various tissues and cell types by quantitative reverse-transcription PCR (qRT-PCR) (Fig. S1 and Fig. 1*B*). In wild-type (WT) cells, *Bcl2l12* mRNA was detectable in all of the tissues tested, among which embryonic fibroblasts, lymphoid organs, lung, and colon expressed relatively higher levels compared with other tissues. As expected, the *Bcl2l12* transcripts were undetectable in *Irf3*$^{-/-}$-derived tissues when the PCR primers were designed to target its 2nd exon (denoted as Primers A, spanning nucleotides 18–63 from the transcription start site) (Fig. S1). When primers corresponding to an intact region (denoted as Primers B, spanning nucleotides 326–405 from the transcription start site) were subjected to the same analysis, low but detectable levels of the *Bcl2l12* transcripts were observed in *Irf3*$^{-/-}$ cells (Fig. 1*B*), presumably owing to the expression of RNA(s) caused by the neomycin-resistant gene cassette. Because no antibody is available for the detection of mouse Bcl2L12, we cannot strictly rule out the possibility that these transcripts would give rise to the expression of some proteins; however, even if the case, the expression would be very low and unlikely to be functional.

Reassessment of IRF3 for its Role in the Activation of the Type I IFN Response. The above findings have formally raised the issue of whether the defect in *Irf3*$^{-/-}$ (and therefore *Bcl2l12*$^{-/-}$) cells described previously (3, 11–14) is because of the loss of IRF3 or Bcl2L12 or both. In this context, it is worth recalling that the ectopic expression of IRF3 in *Irf3*$^{-/-}$*Irf9*$^{-/-}$ MEFs restored their capability to induce the *Ifnb* gene upon infection of Newcastle disease virus (3). This induction is now known to be activated by the cytosolic RNA sensor RIG-I (15), therefore indicating retrospectively that IRF3 is indeed required and sufficient for the activation of the RIG-I-type I IFN pathway. Thus, we expanded our study to examine the contribution of IRF3 in the induction of type I IFN genes following stimulation by a double-stranded RNA poly (rI:rC) and a B-form DNA, poly(dA-dT) · poly(dT-dA) (hereafter referred to as B-DNA), which respectively activates another cytosolic RNA sensor MDA5 (16) and cytosolic DNA sensors (12, 13).

We expressed HA-tagged IRF3 or YFP-tagged Bcl2L12 in *Irf3*$^{-/-}$*Bcl2l12*$^{-/-}$ MEFs by retroviral gene transfer (Fig. S2), and stimulated these cells with poly (rI:rC) or B-DNA and then measured the induction of type I IFN gene mRNAs by qRT-PCR analysis. As shown in Fig. 2 and Fig. S3, the introduction of IRF3 fully restored the defect in the induction of *Ifnb* and *Ifna4* mRNAs in *Irf3*$^{-/-}$*Bcl2l12*$^{-/-}$ MEFs upon stimulation with either poly (rI:rC) or B-DNA. On the other hand, the introduction of Bcl2L12 had no effect on the induction of these genes. Interestingly, the coexpression of Bcl2L12 with IRF3 caused a significant reduction of the mRNA induction levels, possibly owing to the proapoptotic or other functions of Bcl2L12 that may negatively affect the innate immune response. These results can be interpreted to indicate that, as in the case of RIG-I-mediated activation of type I IFN genes, IRF3 is solely responsible and sufficient for the gene activation mediated by MDA5 or as yet unidentified cytosolic DNA sensor(s).

Role of Bcl2L12 in DNA Damage-Induced Apoptosis. Both IRF3 and Bcl2L12 have been implicated in the regulation of apoptosis, but their actual contributions still remain to be determined. However, the role of IRF3 has not been rigorously examined and it is somewhat controversial whether Bcl2L12 is proapoptotic or antiapoptotic (8, 10). Thus, our *Irf3*$^{-/-}$*Bcl2l12*$^{-/-}$ mice provided

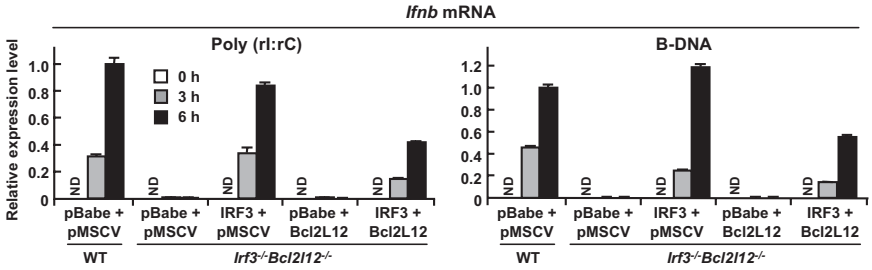

Fig. 2. An essential role of IRF3, but not Bcl2L12, in innate immune responses. Primary MEFs from WT and *Irf3*$^{-/-}$*Bcl2l12*$^{-/-}$ mice were transduced with empty retrovirus, pBabe-HA-IRF3 and/or pMSCV-Bcl2L12-YFP, and lipofected with poly(rI:rC) (10 μg/mL) or B-DNA (10 μg/mL) for the indicated periods. Expression levels for *Ifnb* mRNA were determined by qRT-PCR. The values represent the mean ± standard deviation in triplicate determinations. Data were reproduced in additional 2 experiments.

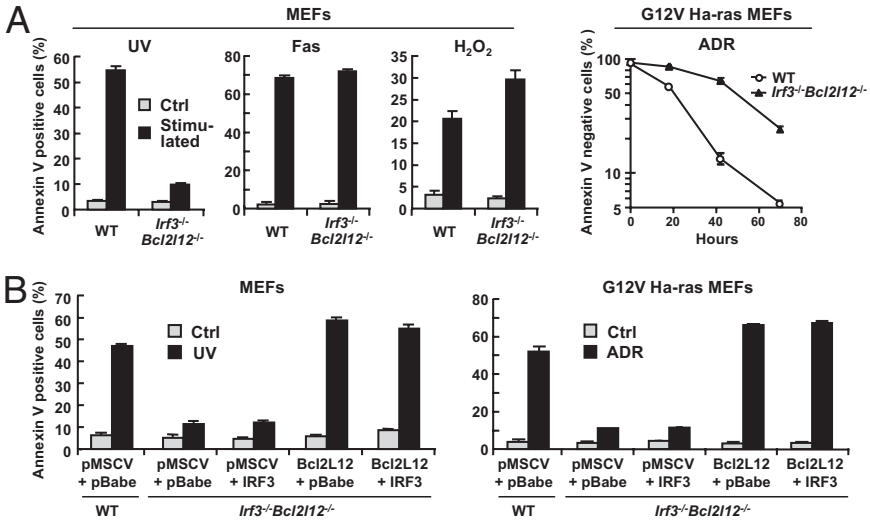

Fig. 3. Sensitivity to apoptotic stimuli in WT and *Irf3*$^{-/-}$*Bcl2l12*$^{-/-}$ cells. (*A*) Primary MEFs and G12V Ha-ras-transduced MEFs. Cells from WT and *Irf3*$^{-/-}$*Bcl2l12*$^{-/-}$ mice were treated with UV (200 J/m^2), an agonistic Fas antibody (100 ng/mL Jo2 monoclonal antibody plus 100 ng/mL protein A in the presence of 1 μg/mL cycloheximide), H_2O_2 (1 mM), or ADR (4 μg/mL). The control (Ctrl) treatment for Fas stimulation was cycloheximide alone. Cells were subjected to annexin V staining at 18 h (primary MEFs) or at the indicated time points (Ha-ras-transduced MEFs). The values represent the mean ± standard deviation in triplicate determinations. Data are representative of at least 3 independent experiments with similar results. (*B*) A proapoptotic role of Bcl2L12. Primary MEFs transduced with HA-IRF3 and/or Bcl2L12-YFP with or without further transduction with G12V Ha-ras were given the indicated apoptotic stimuli for 18 h as in *A* and stained with annexin V. The values represent the mean ± standard deviation in triplicate determinations. Data were reproduced 2 more times.

us with the opportunity to examine the contributions of IRF3 and Bcl2L12 in the regulation of apoptosis.

We first examined MEFs from WT and *Irf3*$^{-/-}$*Bcl2l12*$^{-/-}$ mice in response to 3 types of apoptotic stimulus: DNA damage by UV irradiation, death receptor ligation by an agonistic anti-Fas/CD95 monoclonal antibody, and oxidative stress by H_2O_2 (Fig. 3*A*, *Left*). Interestingly, annexin V staining analysis revealed that *Irf3*$^{-/-}$*Bcl2l12*$^{-/-}$ MEFs are resistant to apoptosis 18 h after stimulation by UV; however, the MEFs are as sensitive as WT MEFs when stimulated by anti-Fas antibody, or treated with H_2O_2. These observations were also confirmed by Hoechst staining (Fig. S4). However, the γ-irradiation-induced cell cycle occurred normally in *Irf3*$^{-/-}$*Bcl2l12*$^{-/-}$ MEFs (Fig. S5). We next expressed an active form of Ha-ras (G12V) in WT and *Irf3*$^{-/-}$*Bcl2l12*$^{-/-}$ MEFs to examine the apoptotic response induced by treatment with the anti-cancer drug, adriamycin (ADR), in these cells (17). As shown in Fig. 3*A* (*Right*), the Ha-ras-expressing *Irf3*$^{-/-}$*Bcl2l12*$^{-/-}$ MEFs showed resistance to apoptosis in response to ADR treatment.

We next examined the contributions of IRF3 and Bcl2L12 to the DNA damage-induced apoptosis of MEFs and Ha-ras-expressing MEFs using the same cells with which we examined the contributions of these molecules in type I IFN gene induction as described above. As shown in Fig. 3*B*, the expression of Bcl2L12 fully restored the defect in the DNA damage-induced apoptosis found in *Irf3*$^{-/-}$*Bcl2l12*$^{-/-}$ MEFs, whereas a similar expression of IRF3 showed no effect. Furthermore, the added expression of IRF3 in Bcl2L12-expressing cells did not show any effect. These observations are in sharp contrast to the results obtained for the type I IFN gene induction showing the requirement of IRF3 but not Bcl2L12, and revealed the critical role of Bcl2L12 but not IRF3 in the DNA damage-induced apoptosis of MEFs.

When we examined thymocytes, however, WT and *Irf3*$^{-/-}$*Bcl2l12*$^{-/-}$ cells showed equivalent apoptotic responses to DNA damage (X-ray and ADR), Fas/CD95 and dexamethasone (Dex) (Fig. S6), despite the relatively high expression level of the *Bcl2l12* transcript in WT thymus (Fig. 1*B* and Fig. S1). These results suggest that thymocytes do not rely on either IRF3 or Bcl2L12 to undergo apoptosis. In this context, it is also worth mentioning that Bcl2L12 did not augment the apoptotic response to Fas ligation (Fig. S7), further confirming the trigger-specific involvement of Bcl2L12 in apoptosis. Thus, while it is clear that Bcl2L12 is crucial to the apoptotic response in MEFs in response to UV irradiation or ADR, the Bcl2L12 pathway appears to be selective in the sense that it is not essential for MEFs in response to other apoptotic stimuli or the apoptotic response of thymocytes.

Bcl2L12-Dependent Activation of Caspases. To gain insight into the DNA damage-induced apoptosis that involves Bcl2L12, we examined the activation status of caspases known to participate in the apoptotic response. Western blot analysis of cell lysates from UV-irradiated WT and *Irf3*$^{-/-}$*Bcl2l12*$^{-/-}$ MEFs revealed that caspase-9 and -3, both of which are known to be critical to the apoptotic response elicited by DNA damage or other stimuli (18, 19), are cleaved in WT MEFs after UV irradiation, but the cleavage of these caspases is strongly suppressed in *Irf3*$^{-/-}$*Bcl2l12*$^{-/-}$ MEFs (Fig. 4*A*). We also found that caspase-2 cleavage occurring in WT cells was also suppressed in *Irf3*$^{-/-}$*Bcl2l12*$^{-/-}$ MEFs (Fig. 4*A*).

It is interesting to note that caspase-2 shows a unique localization pattern in that it is expressed in the nucleus, cytoplasm and the Golgi apparatus (20, 21), and previous reports indicated that this caspase is critical to DNA damage-induced apoptotic response (21–23). Indeed, we also found that the inhibitor specific for caspase-2 suppressed UV-induced apoptosis in WT MEFs (Fig. S8). We next examined the subcellular localization of Bcl2L12 by expressing YFP-tagged Bcl2L12 in *Irf3*$^{-/-}$*Bcl2*$^{-/-}$ MEFs followed by confocal microscopy analysis. As shown in Fig. 4*B*, Bcl2L12 tagged by YFP at its carboxy-terminal region (Bcl2L12-YFP), detected in both the cytoplasm and nucleus, was notably concentrated in the perinuclear region. We then used fluorophore boron dipyrromethene difluoride (BODIPY)-ceramide (24) and MitoTracker, which respectively stain the Golgi apparatus and mitochondria. As shown in Fig. 4*B*, Bcl2L12-YFP was found to merge with BODIPY-ceramide but not with MitoTracker, indicating that Bcl2L12 localizes preferentially in the Golgi apparatus. Clearly, further work will be

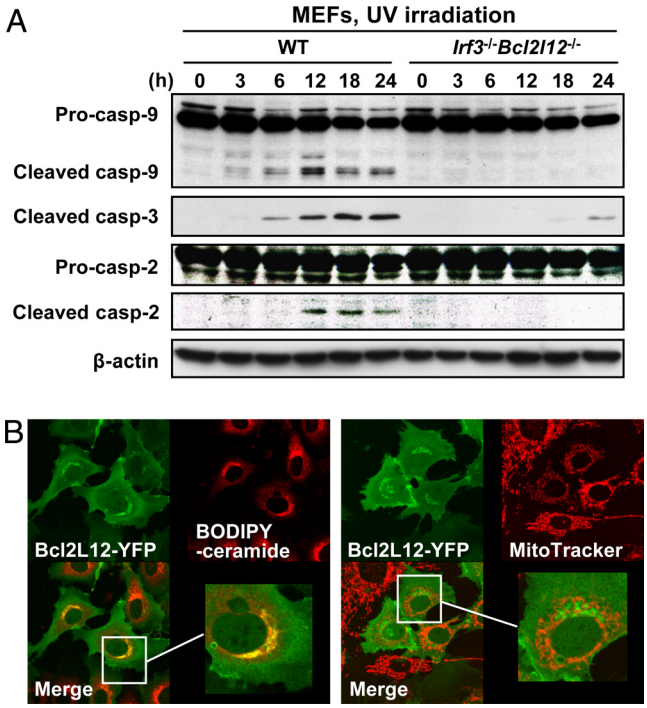

Fig. 4. Impaired cleavage of caspases in $Irf3^{-/-}Bcl2l12^{-/-}$ MEFs and the localization of Bcl2L12. (*A*) Western blot analysis for caspases. WT and $Irf3^{-/-}Bcl2l12^{-/-}$ MEFs were UV irradiated as in Fig. 3 and subjected to western blot analysis at the indicated time points. (*B*) Localization of Bcl2L12. pMSCV-Bcl2L12-YFP was expressed in $Irf3^{-/-}Bcl2l12^{-/-}$ cells and examined by confocal microscopy. BODIPY-ceramide and MitoTracker were used to stain the Golgi membranes and mitochondria, respectively (Original magnification, 600×).

required to clarify the significance of the predominant localization of Bcl2L12 in the Golgi apparatus but, in view of the previous reports indicating the role of caspase-2 in apoptosis (21–23), our study may provide an interesting link between the proapoptotic function of Bcl2L12 and caspase-2 for the cell type-dependent, DNA damage-induced apoptotic response (see *Discussion*).

Discussion

Our present study provides evidence that the products of 2 juxtaposed genes, IRF3 and Bcl2L12, exhibit distinct functions in the regulation of innate immunity and apoptosis. IRF3 has been extensively studied in the context of the activation of type I gene transcription, and the present results together with our previous report (3) confirm that IRF3 is indeed responsible for the activation of the type I IFN response activated by cytosolic RNA or DNA sensors in the immune system. IRF3 has been implicated in the apoptosis induced by DNA damage and virus infection (5, 6, 25). However, our results in this study and previous study on MEFs infected by vesicular stomatitis virus (26) indicate that while IRF3 is indeed critical to the activation of type I IFN genes, its role in apoptosis (and cell cycle arrest) is minimal. In a strict sense, however, a role of IRF3 in the apoptotic response in other cell types or death signaling cannot be ruled out from our present study.

The role of Bcl2L12 in apoptosis has been studied only recently and is still not completely understood. Overexpression experiments have shown that human Bcl2L12 inhibits genotoxic stress- and TNFα-induced apoptosis in primary cortical astrocytes from $Ink4a^{-/-}$ mice (8). Knockdown experiments in human glioblastoma (astrocytic tumor) cell lines also resulted in the same conclusion (8). These data are consistent with the fact that glioblastoma cells highly express *BCL2L12* and are refractory to cancer therapies owing to a marked resistance to apoptosis. In human breast cancer patients, however, *BCL2L12* expression is associated with a favorable prognosis (27), and it has been reported that Bcl2L12 functions as a proapoptotic factor in breast cancer cell lines upon cisplatin treatment (10). Our results obtained using $Irf3^{-/-}Bcl2l12^{-/-}$ MEFs and gene transfer approach clearly show a proapoptotic role of Bcl2L12 upon genotoxic stress, but not death receptor ligation or oxidative stress. It should also be mentioned that human Bcl2L12 has an additional 84 amino acids at the N terminus compared with mouse Bcl2L12 (7). Therefore, the possibility is not rigorously excluded that the seemingly contradictory data between previous studies and our study may be because of a species-specific functional difference between the two Bcl2L12 isoforms.

Our results also imply a link between Golgi-associated Bcl2L12 and caspase-2 and the caspase-9, 3-mediated apoptotic responses. In this context, it has been reported recently that $Casp2^{-/-}$ MEFs, either primary cells or those transduced with Ha-ras and E1a, show resistance to apoptosis triggered by γ-irradiation or several chemotherapeutic drugs (22, 23), whereas $Casp2^{-/-}$ thymocytes undergo apoptosis normally in response to Fas, Dex or γ-irradiation (28). These observations are reminiscent of our present data with $Irf3^{-/-}Bcl2^{-/-}$ mice, and in fact, we found that the cleavage of caspase-2 in response to genotoxic stress is abolished in $Irf3^{-/-}Bcl2^{-/-}$ cells. In addition, we observed that an inhibitor specific for caspase-2 indeed suppressed UV-induced apoptosis in WT MEFs (Fig. S8). Although it still remains unknown how Bcl2L12 regulates the activation of caspase-2, these results imply an interesting possibility that Bcl2L12 and caspase-2 may act as critical components for the interorganelle dialogue between the Golgi apparatus and mitochondria in genotoxic stress-induced apoptosis. Thus, although intriguing, it still remains to be examined how our present study on the role of Bcl2L12 integrates with the well-established mitochondria-dependent apoptotic pathway and whether the Golgi-associated Bcl2L12 functions in the cell- and stimulus-dependent apoptotic response. Clearly, these are future issues to be rigorously addressed.

Finally, in view of the fact that $Irf3^{-/-}Bcl2l12^{-/-}$ mice are currently widely used, our present study may be of wide interest because it confirms that IRF3 is indeed the critical transcription factor for the activation of the type I IFN response by cytosolic RNA and DNA sensors and it also provides compelling evidence that Bcl2L12, but not IRF3, actually contributes to some forms of apoptotic response.

Materials and Methods

Mice and Cells. $Irf3^{-/-}$ mice (3) in the C57BL/6 genetic background and their WT littermates were used for experiments. MEFs and thymocytes were prepared following standard procedures.

Reagents and Instruments. B-DNA, ADR, and Dex were purchased from Sigma–Aldrich, poly(rI:rC) from Amersham Biosciences, and anti-Fas/CD95 antibody (Jo2) from BD PharMingen, z-VAD-FMK from Peptide Institute Inc., and z-VDVAD-FMK from BioVision. UV at 254 nm and X-ray irradiation were performed using UV cross-linker CL-100 (UVP) and X-ray irradiator MBR-1505R2 (Hitachi Medico), respectively.

RNA Analysis. Total RNA was prepared using RNAiso reagent (Takara) followed by RNeasy (Qiagen) according to the manufacturers' instructions. RT was performed using PrimeScript reverse transcriptase (Takara) and oligo dT primer. qRT-PCR was performed using a LightCycler and a SYBRGreen system (Roche). Data were normalized with the level of *Gapdh* expression in each sample. Primer sequences for PCR are available upon request. Microarray analysis was performed using mouse 3D-Gene Mouse Oligo chip 24k (Toray).

Retrovirus Production and Transduction. The retrovirus vector pBabe-HA-IRF3-puro was described in ref. 3. *Bcl2l12* cDNA was amplified by RT-PCR using

PfuUltra DNA polymerase (Promega) and total RNA from WT MEFs, and cloned into pMSCV-YFP-puro, which was generated by inserting EYFP cDNA into pMSCV-puro (Clontech). Retroviruses were produced by transient transfection of 293 EbnaT cells with a retrovirus vector together with pCL-Eco (encoding gag, pol, and an ecotropic envelope protein, purchased from Imgenix) using Lipofectamine 2000 (Invitrogen). MEFs were transduced with retroviruses for 4 h in the presence of 8 μg/ml polybrene (Sigma). Transduced cells were selected by puromycin treatment (1.5 μg/mL) for 3 days. Transduction efficiency was routinely >90%.

Western Blot Analysis, Annexin V Staining, and DNA Content Analysis. Immunoblot analysis was carried out by standard methods. The antibodies used were: anti-hemagglutinin (HA; clone 12CA5) and anti-GFP from Roche; anti-caspase-9 (clone 5B4) from MBL; anti-caspase-3 (Asp-175) from Cell Signaling Technology; anti-caspase-2 (Clone 10C6) from Millipore; and anti-β-actin from Sigma. Staining with annexin V conjugated with FITC or APC (BioVision and BD PharMingen, respectively) was performed as according to the manufacturers' instructions. For DNA content analysis to determine cell cycle distribution and apoptotic (subG$_1$ phase) cells, ethanol-fixed cells were stained with propidium iodide (PI) as described in ref. 29. Stained cells were acquired on a FACSCalibur (BD Biosciences) and data were analyzed using FlowJo software (TreeStar).

Confocal Microscopy. Nuclei were stained with Hoechst 33342 (Invitrogen), the Golgi apparatus with BODIPY TR ceramide (Invitrogen), and mitochondria with MitoTracker Deep Red 633 (Invitrogen) according to the manufacturers' instructions. The cells were viewed on Fluoroview FV1000 (Olympus).

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